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Effect of thymoquinone on micronucleus frequency and proinflammatory cytokine secretion in rat liver on high-fat cholesterol diet

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This study investigates the effects of thymoquinone on micronucleus frequency and proinflammatory cytokine secretion in the livers of rats fed high-fat diet with cholesterol. Thirty-two male *Sprague-Dawley* rats were divided into four groups: control, high-fat diet with cholesterol (CHFD), high-fat diet with cholesterol+thymoquinone (CHFD+T), and thymoquinone (TQ). Histopathological examination revealed hepatocyte vacuolization in the CHFD group. In the CHFD+T group, both hepatocyte vacuolization and mild portal fibrosis were observed, whereas the TQ group exhibited only sinusoidal dilatation. Immunohistochemical analysis showed severe immunoreactivity for IL-6, IL-1β, and TNF-α in the control group, while no such immunoreactivity was detected in the CHFD group. Additionally, in the thymoquinone-treated group, IL-6 and TNF-α displayed moderate immunoreactivity, whereas IL-1β was not observed. In terms of genotoxicity, the CHFD was found to increase the number of micronucleated polychromatic erythrocytes (MNPCEs). However, thymoquinone treatment significantly reduced these elevated levels of MNPCEs. Furthermore, the ratio of polychromatic to normochromic erythrocytes (PCE/NCE), which was lowest in the CHFD group, increased following thymoquinone administration. These results suggest that, with appropriate timing and dosage, thymoquinone may be effective in mitigating liver and genotoxic damage induced by a high-fat diet with cholesterol.

Keywords: Cholesterol, High fat diet, Thymoquinone, Micronucleus, Liver

Consumption of a high-fat diet is a primary factor contributing to obesity and metabolic dysfunctions, including chronic low-grade inflammation[1]. Additionally, high-fat diet intake promotes the accumulation of adipose tissue, which is considered to be a highly active endocrine tissue capable of secreting a variety of factors, including growth factors, cytokines, and hormone-like molecules[2]. Obesity-induced fatty liver often progresses to inflammation and fibrosis. Besides, non-alcoholic steatohepatitis (NASH) is driven by oxidative stress, inflammatory cytokines, such as TNF-α and IL-6, adipokines, mitochondrial dysfunction, and hormones, such as adiponectin and leptin[3]. Similar to alcoholic liver disease, histological findings such as hepatocytes ballooning, inflammatory infiltration, and fibrosis are observed along with fatty liver in NASH[4].

Nigella sativa, popularly known as black cumin, and many of the therapeutic properties of this plant are associated with thymoquinone, the primary bioactive component of the essential oil it contains[5]. Thymoquinone has been extensively investigated in numerous studies due to its potent antioxidant, anti-inflammatory, antimicrobial, and hepatoprotective qualities[5-7].

Tumour necrosis factor-alpha (TNF-α), interleukin (IL-1) and IL-6 are cytokines implicated in the pathophysiology of obesity, type 2 diabetes, arterial hypertension, and metabolic disorders are all affected by proinflammatory cytokines. Furthermore, proinflammatory cytokines have been reported to play a significant role in the development of liver diseases[8,9].

The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in bone marrow preparations is used to predict any deterioration in haematopoiesis caused by the administered substance[10]. The decrease in the PCE/NCE ratio indicates that the administered substance penetrates the bone marrow

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and exerts a toxic effect by inhibiting the proliferation and maturation of nucleated precursor erythrocyte cells, hence decreasing erythrocyte formation[11].

The aim of this study is to investigate the effect of thymoquinone on micronucleus frequency and proinflammatory cytokine secretion in the liver of rats fed cholesterol high-fat diet.

Materials and Methods

Ethics statement

The study protocol was approved by Laboratory Animals Ethics Committee of Kafkas University (KAU-HADYEK:2021/018) for this study.

Experimental design

The experimental animals used in this study were supplied from the Laboratory Animals Unit of Atatürk University. Thirty-two 40-day-old male *Sprague-Dawley* rats weighing 265-275 g that have not been used before in any study were used. The rats were housed at a temperature of 25±2°C with a humidity of 60-65% and a 12-hour light/dark cycle, in cages that were cleaned daily and they fed were *ad libitum* with rat feed, with four animals per cage. The live weights of randomly selected rats were measured, and the following groups were created. Control group (n=8): No treatment was applied to this group of rats; High-fat diet with cholesterol (CHFD) group (n=8):For eight weeks, rats in this group were fed pellet feed mixed with butter and 2% cholesterol at the rate of 65% of their daily energy intake[12]; High-fat diet with cholesterol and thymoquinone (CHFD+T) group (n=8): The rats, which were fed in the same manner as the rats fed in the CHFD group for eight weeks, were administered 8 mg/kg thymoquinone (Sigma-Aldrich/274666-5G) that was dissolved in physiological saline intraperitoneally for 14 days following the end of the eighth week[13]; Thymoquinone (TQ) group (n=8):Animals that were fed standard pellet feed for 8 weeks were administered 8 mg/kg of thymoquinone that was dissolved in physiological saline intraperitoneally for 14 days[13].

After measuring the body weight of the rats in each group, cervical dislocation was performed under anesthesia, and liver tissues were taken for histology and immunohistochemistry. In addition, femur bones were taken for the micronucleus frequency.

Histopathological examination

After fixing the liver tissue samples in a 10% formaldehyde solution, they were blocked in paraffin after routine histological procedures. To demonstrate the general structure of the tissue, 5 μm sections taken from blocks were stained by hematoxylin and eosin (H&E) staining technique.

Immunohistochemical examination

The immunoreactivity of IL-1β, IL-6 and TNF-α was determined in liver tissue using the streptavidin-biotin peroxidase method (Hsu *et al.*). For 15 minutes, 5 μm thick sections taken from paraffin blocks were kept in a solution of hydrogen peroxide in methanol (3%) to inhibit endogenous peroxide activity. After deparaffinization and dehydration, the sections were placed in citrate buffer solution (pH 6.0) and boiled in the microwave for 10 minutes at 800 watts to expose antigenic receptors. After that, it was incubated using a large volume ultra V block solution for 10 minutes. The sections were subjected to IL-1β (1/500) (Santa Cruz, sc52012), IL-6 (1/500) (Biorbty, orb651448) and TNF-α (1/500) (Santa Cruz, sc52746) antibodies diluted in PBS for 1 hour at room temperature. Then, it was incubated for 15 minutes at room temperature with Biotinylated Goat Anti-B Polyvalent solution and Streptavidin peroxidase solution, respectively. A chromogen solution containing 3.3-diaminobenzidine tetrahydrochloride (0.5 mg DAB/mL, Dako Corporation, Carpinteria, USA) that was dissolved in a phosphate buffer solution was injected into the sections for 3 minutes. After counter staining with modified Gill III haematoxylin, the sections were dehydrated, immunomounted, and examined and photographed under a light microscope (Leica DM4000B, Germany).

The percentage of stained cells and the degree of staining were used as criteria in the sections, and scoring was accomplished using a semi-quantitative method. Immunohistochemical examinations were performed by checking whether or not the target cells were stained and the intensity of staining in the stained target cells. Two independent observers evaluated them by giving scores ranging from 0 to 3 based on the features of no staining (0), weak staining (1), moderate staining (2), and strong staining (3).

Detection of micronucleus frequency

Micronucleus testing was run on femur bone in the study. The femur bone was sliced on both ends and placed in a bone marrow injector with a centrifuge tube containing 3 mL of calf serum. Supernatants were discarded after centrifuging tubes containing bone marrow samples at 2000 rpm for 5 minutes. It was subsidized by adding a drop of calf serum to the

remaining portion in the tube. On clean slides, a drop of that sample was smeared. After smearing, the slides were air dried and fixed with methyl alcohol for 10 minutes. The fixed preparations were stained for 5 minutes with 0.25% May Grunwald stain and rinsed with distilled water. It was then re-stained for 5 minutes using 0.125% May Grunwald stain and rinsed in distilled water. Finally, it was stained with 20% Giemsa stain for 30 minutes, rinsed and left to dry. The preparations were magnified 1000 times under an Olympus CX21 light microscope and 2000 PCEs were counted randomly from each preparation. The percentages of micronucleated polychromatic erythrocytes (MNPCE) were calculated by determining their number among them. Also, PCE and NCE were counted in 1000 randomly selected erythrocytes from each preparation, and the PCE/NCE ratio was calculated statistically and their percentages were determined. Schmid (1975) was the first to develop bone marrow preparations, which were then prepared using a method adapted to our laboratory and working conditions[10].

Statistical analysis

The SPSS 18 (IBM Corp., New York, USA) package program was used to analyse the data. The normality and One-Way ANOVA tests were applied in order to identify the differences between the groups. To compare significant differences between groups, Duncan′s test was utilised.

Table 1 — Statistical evaluation of live weight (g) results between the groups

Week	Control	CHFD	CHFD+T	TQ	P
0	265.89[a]	269.39[a]	273.34[a]	267.62[a]	0.971
10	421.37[b]	479.25[a]	467.62[a]	397.37[b]	0.001

[[a, b]Different letters in the same column indicate statistical significance (P<0.05)]

Results

Live weights results

A significant difference was observed in live weights of the groups before and after the experiment (P<0.05) (Table 1).

Histopathological results

Histopathological examination revealed a normal hepatic architecture in the control group. However, hepatocyte vacuolization was observed in the liver tissue of the CHFD group. In the CHFD+T group, hepatocyte vacuolization was accompanied by mild fibrosis in the portal areas. In contrast, the TQ group exhibited only sinusoidal dilatation (Fig. 1).

Immunohistochemical results

Immunohistochemical analysis revealed distinct staining patterns across groups (Table 2).

IL-6: Hepatocellular immunoreactivity was strong in controls but weak to absent in all treatment groups. In sinusoidal walls, expression was strong only in the

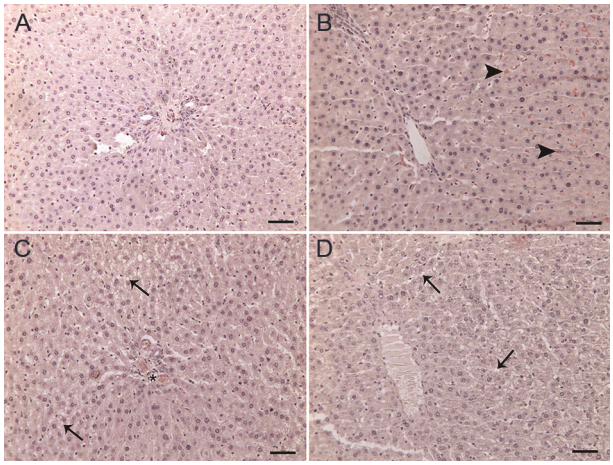

Fig. 1 — Rat liver tissue (A) Control group, (B) TQ group, (C) CHFD+T group, (D) CHFD group, *: Mild fibrosis, Arrow head: Dilatation in sinusoids, Arrow: Vacuolization, Bar: 50 µm H&E.

Table 2 — Immunohistochemical grading between groups

Groups		Control	CHFD	CHFD+T	TQ
IL-6	Hepatocytes (Mean ± SD)	2.75 ± 0.45	0.00 ± 0.00	0.75 ± 0.45	0.44 ± 0.51
	Hepatocytes Median (Min-Max)	3.0 (2-3)	0.0 (0-0)	1.0 (0-1)	0.0 (0-1)
	Sinusoidal wall (Mean ± SD)	1.06 ± 0.68	0.00 ± 0.00	0.81 ± 0.40	2.75 ± 0.45
	Sinusoidal wall Median (Min-Max)	1.0 (0-2)	0.0 (0-0)	1.0 (0-1)	3.0 (2-3)
IL-1β	Hepatocytes (Mean ± SD)	2.69 ± 0.48	0.13 ± 0.34	0.06 ± 0.25	1.56 ± 0.51
	Hepatocytes Median (Min-Max)	3.0 (2-3)	0.0 (0-1)	0.0 (0-1)	2.0 (1-2)
	Sinusoidal wall (Mean ± SD)	0.19 ± 0.40	0.13 ± 0.34	1.81 ± 0.40	2.75 ± 0.58
	Sinusoidal wall Median (Min-Max)	0.0 (0-1)	0.0 (0-1)	2.0 (1-2)	3.0 (1-3)
TNF-α	Hepatocytes (Mean ± SD)	2.75 ± 0.58	0.06 ± 0.25	1.69 ± 0.48	0.63 ± 0.50
	Hepatocytes Median (Min-Max)	3.0 (1-3)	0.0 (0-1)	2.0 (1-2)	1.0 (0-1)
	Sinusoidal wall (Mean ± SD)	0.38 ± 0.50	0.06 ± 0.25	0.19 ± 0.40	2.19 ± 0.54
	Sinusoidal wall Median (Min-Max)	0.0 (0-1)	0.0 (0-1)	0.0 (0-1)	2.0 (1-3)

TQ group, while remaining weak or undetectable in others (Fig. 2).

IL-1β: Hepatocyte staining was strong in controls and moderate in the TQ group, but undetectable in CHFD and CHFD+T groups. Conversely, sinusoidal reactivity was absent in controls and CHFD but increased to moderate levels in CHFD+T and strong levels in the TQ group (Fig. 3).

TNF-α: Hepatocellular immunoreactivity followed a graded pattern: strong in controls, moderate in CHFD+T, weak in TQ, and absent in CHFD. Sinusoidal reactivity was observed exclusively in the TQ group (moderate), absent in all other groups (Fig. 4).

Additionally, negative control staining was performed to control for non-specific binding and false positive results (Fig. 5).

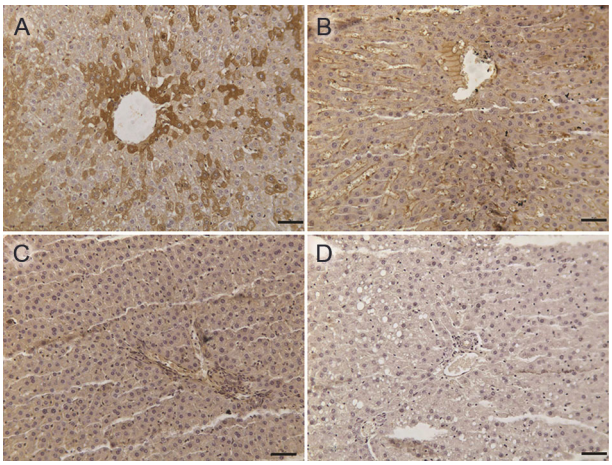

Fig. 2 — IL-6 immunoreactivity in rat liver tissue. (A) Control group, (B) TQ group, (C) CHFD+T group, (D) CHFD group. Bar: 50 µm.

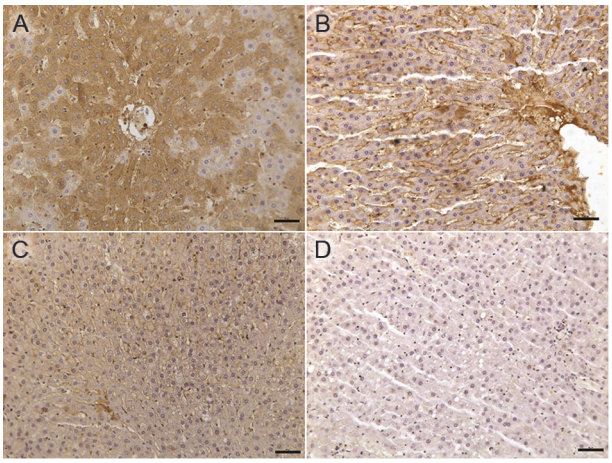

Fig. 3 — IL-1β immunoreactivity in rat liver tissue. (A) Control group, (B) TQ group, (C) CHFD+T group, (D) CHFD group. Bar: 50 µm.

Cytogenetic results

The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was significantly higher in the CHFD and CHFD+T groups compared to the control group (P<0.001). No significant difference was observed in the MNPCE frequency of the TQ group compared to the control (P>0.05). However, the MNPCE count in the CHFD+T group was significantly lower than that of the CHFD group (P<0.001) (Table 3, Fig. 6 & Fig. 7).

Regarding the PCE/NCE ratio, no statistical difference was found between the control and TQ groups. The CHFD group exhibited the lowest PCE/NCE ratio; however, this ratio significantly increased in the CHFD+T group compared to the CHFD group (Table 3, Fig. 8).

Discussion

Previous studies have shown that feeding with a cholesterol high-fat diet produces hepatocellular

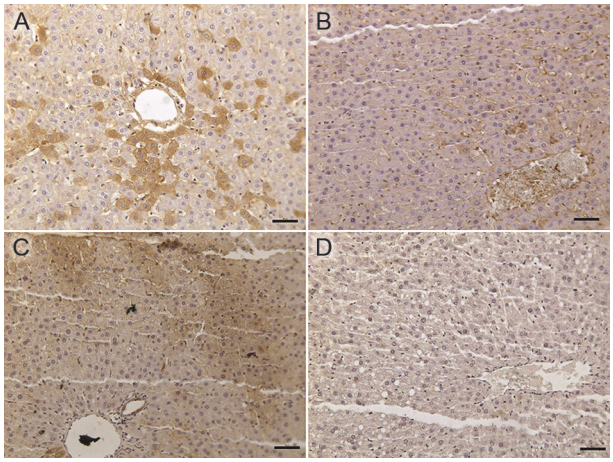

Fig. 4 — TNF-α immunoreactivity in rat liver tissue. (A) Control group, (B) TQ group, (C) CHFD+T group, (D) CHFD group. Bar: 50 µm.

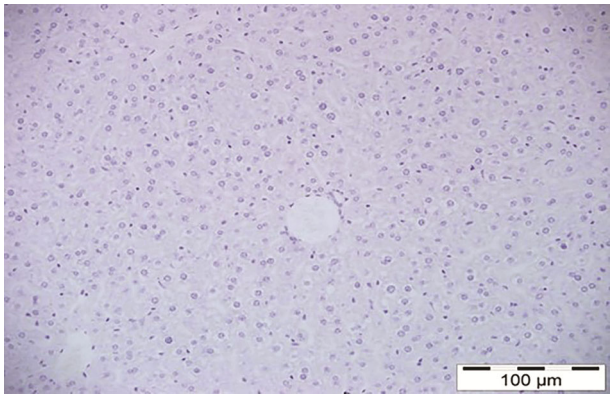

Fig. 5 — Negative control. Bar: 100 µm.

Table 3 — Comparison (%) of the results from the micronucleus test between the control and experimental groups

Groups	Total PCE	MNPCE	MNPCE Rate (%)	Group Average	Total Erythrocyte (PCE+NCE)	Number of PCE	Number of NCE	PCE/NCE Ratio
Control	16000	51	0.31	6.37	8000	5152	2848	1.81
CHFD	16000	393	2.45	49.12	8000	3570	4430	0.8
CHFD+T	16000	175	1.09	21.87	8000	4115	3885	1.06
TQ	16000	53	0.33	6.62	8000	5175	2825	1.83

[PCE: Polychromatic erythrocyte, MNPCE: Micronucleated polychromatic erythrocytes, NCE: Normochromatic erythrocyte]

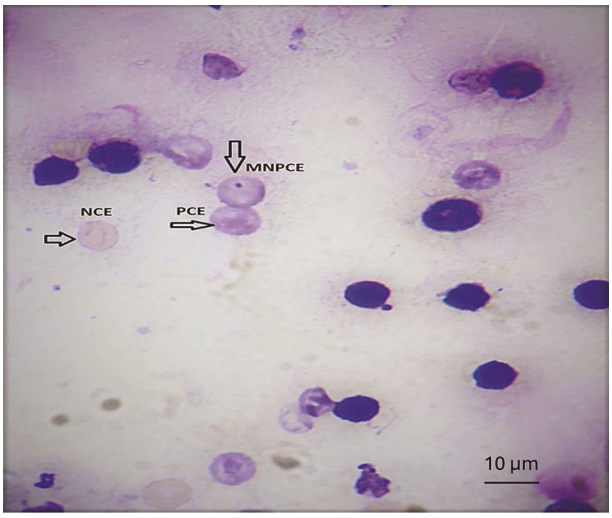

Fig. 6 — Microscopic image of MNPCE, PCE and NCE in bone marrow cells of rats in the CHFD group (×1000).

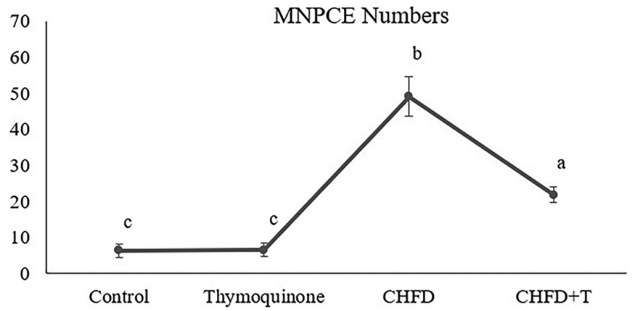

Fig. 7 — MNPCE numbers of control and experimental groups.

steatosis, hepatocyte ballooning, lobular inflammation, and steatohepatitis in rat and mouse liver cells[14,15]. Furthermore, it has been demonstrated that CHFD significantly increases the number of inflammatory foci and macrovesicular steatosis in liver lobules, with a high probability of microvesicular steatosis progression[16]. Hepatic necrosis was reported in specific areas in the liver tissue of rats that are fed a cholesterol high-fat diet, as well as vacuolization in hepatocytes associated with portal infiltration by inflammatory cells[17]. Consistent with these findings, the present study confirmed that the administration of an CHFD caused severe histopathological changes, including hepatocyte vacuolization, in the liver tissue.

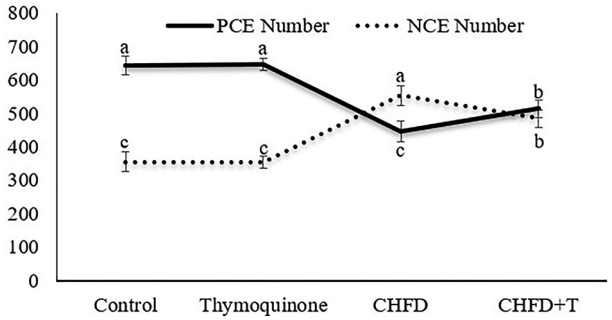

Fig. 8 — PCE and NCE numbers of control and experimental groups.

Several researchers have reported the protective role of many herbs and their active ingredients, including thymoquinone, on liver tissue[14,18]. It was demonstrated that thymoquinone protects against hyperlipidemia-induced hepatic damage, including lobular architectural distortion, focal necrosis, hepatocellular swelling, and widespread lipid droplet accumulation in mice[19]. Similarly, another study reported that thymoquinone administration ameliorated histopathological alterations in mice fed a high-fat diet[20]. The present study revealed vacuolization and mild fibrosis in the portal areas of some hepatocytes in the CHFD+T group, and dilatation only in the sinusoids in the thymoquinone group.

Proinflammatory cytokines, which are excessively secreted into circulation or tissues, play a critical role in various pathogenesis of liver diseases[21]. Kupffer cells and hepatocytes in the liver produce these cytokines, which are involved in lipid metabolism and hepatic inflammation[22]. IL-6 is a cytokine with both proinflammatory and anti-inflammatory effects and transforms monocytes into macrophages[23]. Furthermore, adipocytes and macrophages in the subcutaneous adipose tissue are major sources of IL-6[24]. While no IL-6 secretion was found in the liver tissue of the control group in one study on rats, another study reported IL-6 secretion in the liver perivenular hepatocytes of the control group[25,26]. The present study, similar to the study by Fukumura *et al*. showed that IL-6 secretion was strong in hepatocytes

but weak in the sinusoids in the liver tissue of the control group. It is stated that feeding with a high-fat diet promotes the secretion of IL-6 from liver tissue[19]. However, the present study revealed no IL-6 immunoreactivity in either hepatocytes or sinusoid walls in the cholesterol high-fat diet group. The studies have reported that the intensity of IL-6 secretion, which increases with a high-fat diet or a cholesterol high-fat diet, reduces when a plant extract (caterpillar fungus, squirting cucumber) or an active substance (*Dendrobium officinale*) is used[18,27]. In the present study, contrary to these results, it was observed that thymoquinone increased IL-6 secretion in the CHFD+T group, but that immunoreactivity was quite weak. Also, IL-6 immunoreactivity was weak in hepatocytes and strong in sinusoid walls in groups to which only thymoquinone was administered.

IL-1β, a member of the IL-1 family, plays an important role in proinflammatory reactions and immune regulation in response to pathogen-induced tissue damage[28]. IL-1β immunoreactivity was observed to be strong in liver hepatocytes of the control group in the present study. However, the present study indicated no IL-1β immunoreactivity in hepatocytes in the cholesterol high-fat diet group. It is believed that such difference may be caused by differences in the studies conducted for the evaluation of IL-1β secretion or the regional sample collected from the liver tissue. The administration of thymoquinone was reported to suppress or reduce IL-1β expression in rats fed a high-fat diet[29]. It was argued that the administration of thymoquinone protected tissues by lowering IL-1β in the blood and tissues[17,30]. In contrast to previous studies, IL-1β secretion was observed to be moderate in some sinusoidal walls in the CHFD+T group, but strong in the liver sinusoidal wall of the groups that were administered only thymoquinone, but moderate in some hepatocytes in the present study.

TNF-α is a potent proinflammatory cytokine secreted from myeloid cells by activation of MAPK and NF-κB signalling pathways[31]. It is mostly secreted by macrophages and lymphocytes in response to cellular damage caused by infection or malignant transformation[32]. It is also, secreted by many other cells and tissues, including activated Kupffer cells in the liver. It exacerbates both the inflammatory response and oxidative stress in hepatic cells after secretion[8]. The cells, such as hepatocytes, Kupffer cells, and endothelial cells are activated by TNF-α and reactive oxygen species (ROS)[33]. Its levels rise in both acute and chronic diseases[30].

While a study on rats reported that no TNF-α secretion was found in the liver tissue of the control group[26],. Hunt *et al*.[34] suggested in their study that TNF-α secretion was found in the liver tissue of mice in the control group. The study conducted on human liver cells stated that healthy liver cells secreted a small amount of TNF-α[35]. Orfila *et al.*[36] on the other hand, reported that TNF-α positive cells were rarely identified along the sinusoids in the livers of the control group, but they displayed no staining in hepatocytes. Similar to the findings of Hunt *et al.*[34]and Gonzalez *et al*.[35] TNF-α immunoreactivity was observed in the liver tissues of the control group in the present study. This suggested that TNF-α secretion may vary across healthy liver tissues of different species.

Natural products block the inflammatory mechanisms induced by cytokine secretion and reduce the production of pro-inflammatory factors by macrophages[37]. The existing studies also report that plant extracts and active ingredients reduce TNF-α secretion in liver cells when fed an experimentally high-fat diet or a cholesterol-rich high-fat diet[16,38]. TNF-α secretion in the livers of male/female mice that were fed a short-term high-fat diet did not change. It was reported that TNF-α levels, however, increased roughly twice in the livers of male mice that were fed a long-term high-fat diet compared to the control group, whereas female mice showed no significant changes[39]. Fki *et al*.[40] reported that feeding a high-fat diet significantly increased TNF-α expression.

Feeding both a cholesterol diet and a high-fat diet was reported to increase the secretion of TNF-α in the liver[17,41]. However, the present study revealed no TNF-α immunoreactivity in rats fed a cholesterol high-fat diet. This is considered to be due to the fact that either a long-term feeding with a fatty diet or the difference between species may have an effect on TNF-α secretion in the liver.

Thymoquinone has been indicated in existing studies to reduce proinflammatory cytokines such as interleukins and TNF-α[42]. TNF-α secretion was observed to be moderate in the liver tissue of the CHFD+T group in the present study. Contrary to existing studies, this indicated that the administration of thymoquinone increased TNF-α secretion, which decreased as a result of feeding with a cholesterol

high-fat diet. Also, TNF-α immunoreactivity was determined to be moderate in the sinusoid walls and weak in some hepatocytes as a result of the administration of thymoquinone to rats fed a normal feed. A study on the blocking of TNF-α secretion indicated that autoimmune disorders such as diabetes can develop. Therefore, we believe that a high-fat diet may make the body vulnerable to autoimmune disorders by reducing TNF-α secretion.

Obesity and high cholesterol lead to cellular and molecular changes, including oxidative stress and DNA damage[43]. Studies on humans have reported an increase in the levels of DNA damage in overweight and obese groups compared to normal subjects[44]. A study on obese animals have also indicated that obesity leads to an increase in genetic damage[38]. According to our findings, the rate of MNPCE, a marker of genetic damage, increased significantly in the group that received a cholesterol high-fat diet compared to the other experimental groups, which is compatible with previous studies. When compared to all other experimental groups, the PCE/NCE ratio decreased significantly in the cholesterol high-fat diet group.

Luciano *et al.* reported that the comet assay revealed significant DNA damage in both blood and liver tissues of mice subjected to a high-fat diet. They further demonstrated that *Zingiber officinale* extract effectively mitigated this genotoxicity, highlighting its protective potential against cellular damage[38]. Thymoquinone efficiently kills tumour cells without producing cytotoxicity in normal cells[45]. Also, black cumin had cytoprotective and gene-protective effects and regulated cell proliferation[46]. The study to assess the genotoxic effect of black cumin reported that there was no significant difference in MNPCE levels of the control group and the groups that were subjected to black cumin, and black cumin had no genotoxic effect. The application of black cumin was also revealed to reduce the genetic damage induced by cisplatin[46]. Another study tested the antigenotoxic effect of black cumin oil on hepatotoxicity induced by carbendazim and mancozeb. The study data indicated that black cumin oil reduced the frequency of MN, DNA damage, and frequency of chromosomal aberrations[47]. According to the findings of the study, when compared to the control group and the groups that were administered thymoquinone, it was indicated that a cholesterol high-fat diet caused an elevation in the frequency of MNPCE, but the administration of thymoquinone decreased the frequency of MNPCE, which is compatible with the studies. In the present study cholesterol high-fat diet was observed to cause a significant decrease in the PCE/NCE ratio as well as an increase in the PCE/NCE ratio as a result of the administration of thymoquinone.

Conclusions

In conclusion, the present study demonstrates that a cholesterol high-fat diet (CHFD) causes significant histopathological alterations, specifically hepatocyte vacuolization and leads to genotoxicity as evidenced by increased MNPCE frequency and decreased PCE/NCE ratios. Notably, contrary to the typical inflammatory response, CHFD appeared to suppress the immunoreactivity of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in liver tissue in this experimental model. Crucially, the administration of thymoquinone exhibited a protective potential against these adverse effects. Thymoquinone treatment significantly alleviated the genetic damage induced by the high-fat diet and ameliorated cell proliferation rates. Furthermore, it modulated the cytokine expression profile, tending to partially restore the immune markers suppressed by the dietary stress. These findings suggest that thymoquinone acts as an effective antigenotoxic agent and a potential immune modulator against hepatic stress induced by a cholesterol high-fat diet.

Conflict of interest

The authors have no conflicts of interest to report.

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